Improved 6-Plex TMT Quantification Throughput Using a Linear Ion Trap – HCD MS^3 Scan

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Extended Methods

Sample Preparation.

We used six-plex TMT reagents to build a model peptide mixture sample from protein digests of *Saccharomyces cerevisiae*. The six-plex TMT system allows for quantification in up to six reporter channels through reporter ions at *m/z* 126, 127, 128, 129, 130 and 131. For each of the six reporter channels, 4 µg of tryptic-digested yeast peptides were resuspended in 24 µL of 100 mM triethyl ammonium bicarbonate (TEAB); the pH of the solution was confirmed to be between 8.5-9.0. To this mixture was added 6.6 µL of the TMT reagent, dissolved in acetonitrile (41 µL for 0.8 mg reagent). The reaction was incubated at room temperature for 1 h, protected from light. The reaction was quenched with 1.3 µL 5% hydroxylamine (in 100 mM TEAB), for 15 min, at room temperature, protected from light. The total volume of each reaction was brought to 40 µL with 100 mM TEAB, such that each sample had a peptide concentration of 100 ng/µL. The reactions were mixed to give a 10:2:1:1:2:10 mass ratio:

25 µL (2.5 µg) 126-labeled peptides,
5 µL (0.5 µg) 127-labeled peptides,
2.5 µL (0.25 µg) 128-labeled peptides,
2.5 µL (0.25 µg) 129-labeled peptides,
5 µL (0.5 µg) 130-labeled peptides, and
25 µL (2.5 µg) 131-labeled peptides.

The combined mixture of TMT-labeled peptides was concentrated *in vacuo* and then resuspended in 0.2% formic acid. The peptide mixture was then desalted by HPLC using a C18 Macrotrap (Michrom Bioresources) (buffer A: 0.2% formic acid in H_2O; buffer B: 0.2% formic acid in acetonitrile), followed by concentration *in vacuo*. Based on the UV chromatogram, >99% of starting peptide was retained through the labeling and chromatography process.

Full description of the LC-MS methods

All LC-MS experiments were performed on an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) coupled to an ancillary EASY-nLC II nano-HPLC (Thermo Fisher Scientific) equipped with a Nanospray Flex ion source (Thermo Fisher Scientific). Samples were directly loaded onto a 20-cm, 50 µm I.D. Picrofrit microcapillary column (New Objective), packed in-house with ReproSil-Pur C_{18AQ} 1.9 µm resin (120 Å, Dr. Maisch GmbH, Ammerbuch, Germany). Separation was achieved by applying a 1.6-32% ACN gradient in 0.2% formic acid over 120 minutes at 200 nL/min. Specifically, the gradient was as follows: 2-6% solvent B (7.5 min), 6-25% solvent B (82.5 min), 25-40% solvent B (30 min), 40-100% solvent B (1 min) and 100% B (9 min). Solvent A was 97.8% H_2O, 2% acetonitrile, 0.2% formic acid; solvent B was 19.8% H_2O, 80% acetonitrile, and 0.2% formic acid. The column was enclosed in a column heater operating at 75 °C. Electrospray ionization was enabled by applying a voltage of 2.5 kV through the coated tip.

The Orbitrap Fusion was operated in data-dependent acquisition mode for both the MS^3-IT and MS^3-OT methods. The $FTMS^1$ and $ITMS^2$ settings were the same for both methods. For the $FTMS^1$ spectra, a survey scan of 400-1500 *m/z* was collected in the Orbitrap at a resolution of 120,000, an AGC target of $2x10^5$, and a max injection time of 50 ms. The 10 most intense ions were selected for MS analysis. Precursor ions were filtered according to charge state (2-7), dynamic exclusion (70 s with a ± 10 ppm window), and monoisotopic precursor selection.

MS^1 precursors were first interrogated by $ITMS^2$ using CID (35% collision energy, rapid scan rate, scan range of 400-2000 *m/z*). Precursors were isolated using a 0.5 *m/z* isolation window. They were accumulated to an AGC target of 4,000 or a max injection time of 150 ms. The intensity threshold was $1x10^3$.

For the SPS-MS^3 scan in the **Orbitrap**, parameters included an AGC target of $5x10^4$, max injection time of 250 ms, HCD normalized collision energy of 55, quadrupole isolation width of 2 *m/z*, SPS selection of the 10 most intense $ITMS^2$ fragment ions, an Orbitrap resolution of 60,000, and a scan range of 100-500 *m/z*.

For the SPS-MS^3 scan in the **Ion Trap**, parameters included an AGC target of $1x10^3$-$1x10^4$, max injection time of 250 ms, HCD normalized collision energy of 55, quadrupole isolation width of 2 *m/z*, SPS selection of the 10 most intense $ITMS^2$ fragment ions, rapid scan mode, and a scan range of 100-500 *m/z*.

Data Analysis.
Thermo raw files were converted to MGF files using ReAdW4Mascot2 (v. 20090305a). MS^2 spectra were searched against the SGD yeast database (5,911 sequences) and a contaminant database (259 sequences) using MSGF+ (v. 20140630). Precursor tolerance was 8 ppm. CID was specified as the fragmentation method and "low-res LCQ/LTQ" was specified as the MS^2 mass analyzer. Trypsin was specified as the digestion enzyme. Number of tolerable tryptic termini was 2. Fixed modifications included carboxyamidomethyl of cysteine and TMT6plex modification of peptide N-termini and lysine. Variable modifications included oxidation of methionine and protein N-terminal acetylation. A decoy search was included and PSM and peptide false discovery rates were both less than 1%. MS^3 reporter ion mass tolerances were 0.5 Da for MS^3-IonTrap and 0.02 Da for MS^3-Orbitrap. Reporter ion intensities were corrected for isotope impurities. In-house scripts were used to pair the MS^2 identifications with the MS^3 spectra and to perform protein inference from the peptides identified.

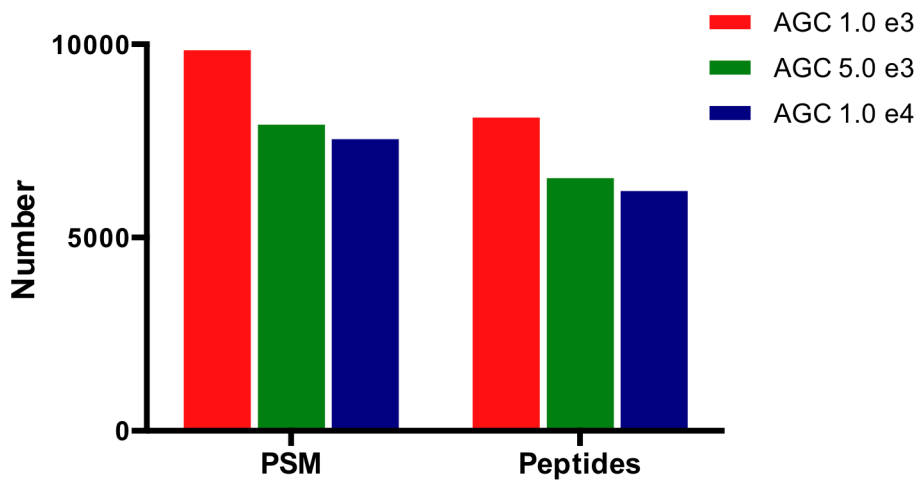

Figure S1. Summary of data for each of the three AGC targets tested for the MS^3-HCD scan when using the linear ion trap for the MS^3 scan. The number of peptide-spectrum matches (PSM) and the number of unique, identified peptides (Peptides) that were quantified in all six channels are shown.

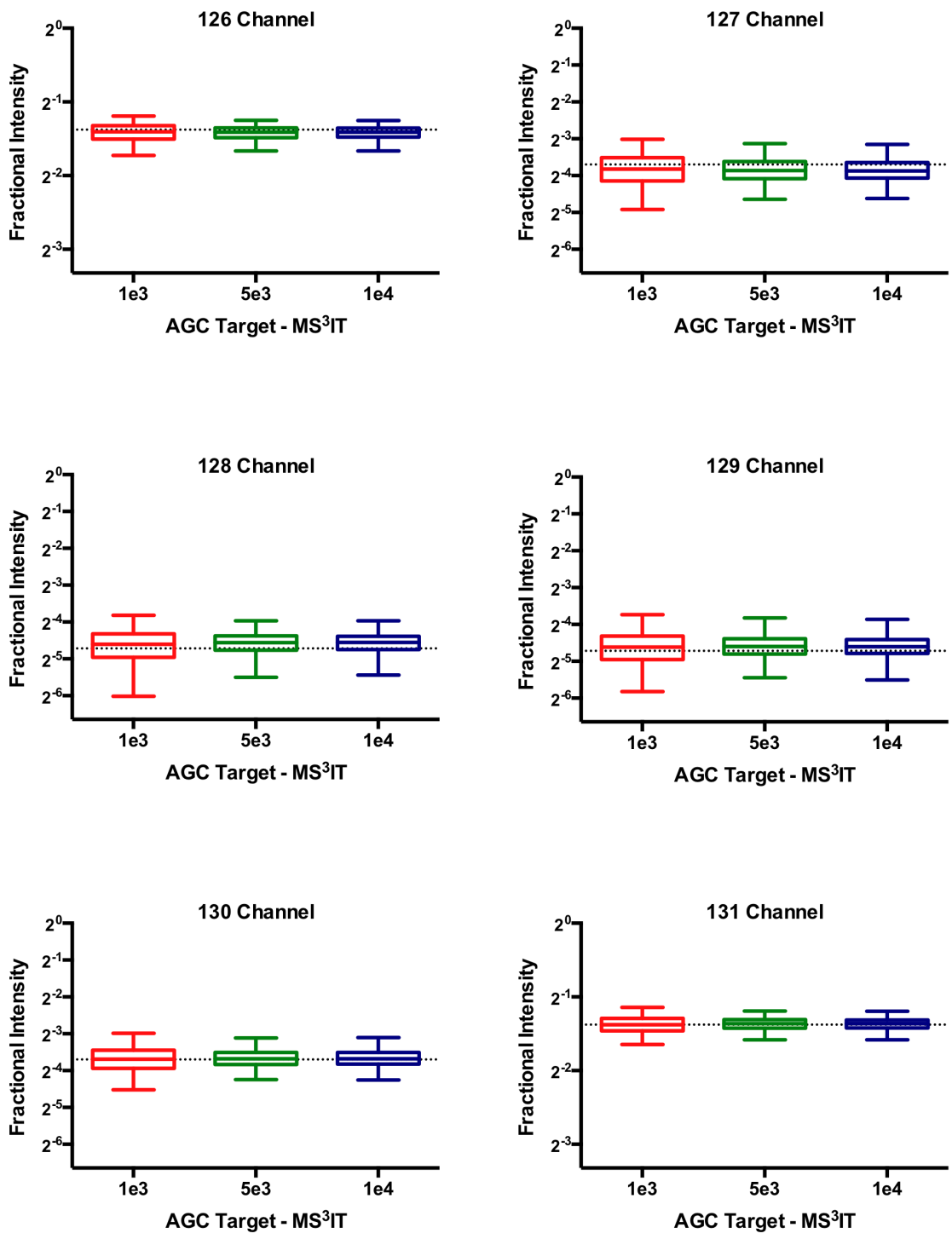

Figure S2. Accuracy and precision of PSM quantification when collecting the MS^3-HCD scans using the linear ion trap with three different AGC targets. Boxplots indicate the median (middle line), the interquartile range (box) and the 5th and 95th percentile (whiskers) for each experiment. Dashed lines indicate the values for the idealized fractional intensities.

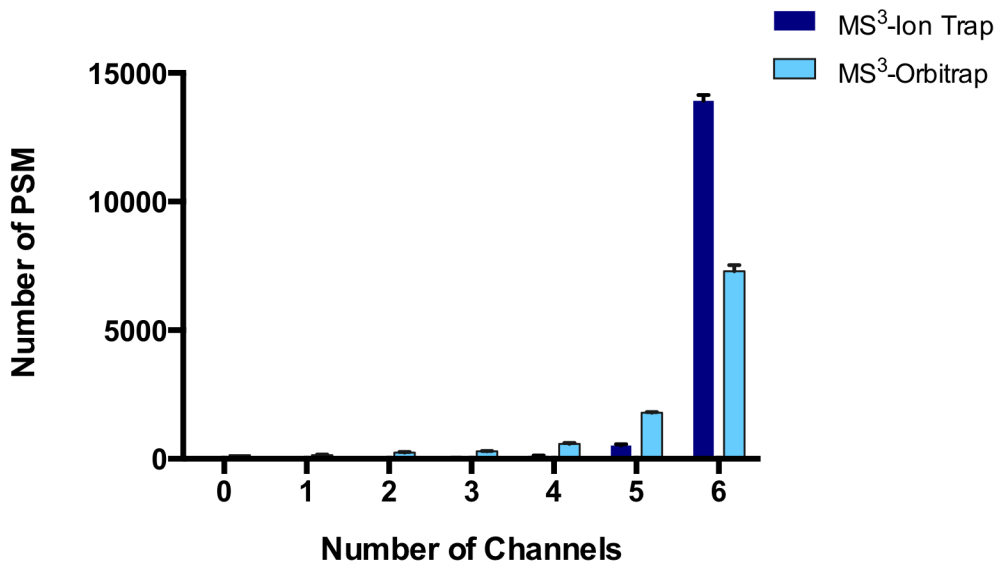

Figure S3. Number of peptide-to-spectrum matches (PSM) that had reporter ions quantified in the indicated amount of reporter channels. For the MS^3 scans collected in the linear ion trap, 95% of the PSM were quantified in all six channels; for the MS^3-Orbitrap scans, 70% of the PSM were quantified in all six channels. For normalization and quantitative downstream data analysis for all samples, it is necessary to have reporter ion intensities observed in all six reporter channels. Thus, we focused on only those PSM that were quantified in all six channels. Error bars represent the standard deviation of five technical replicates.

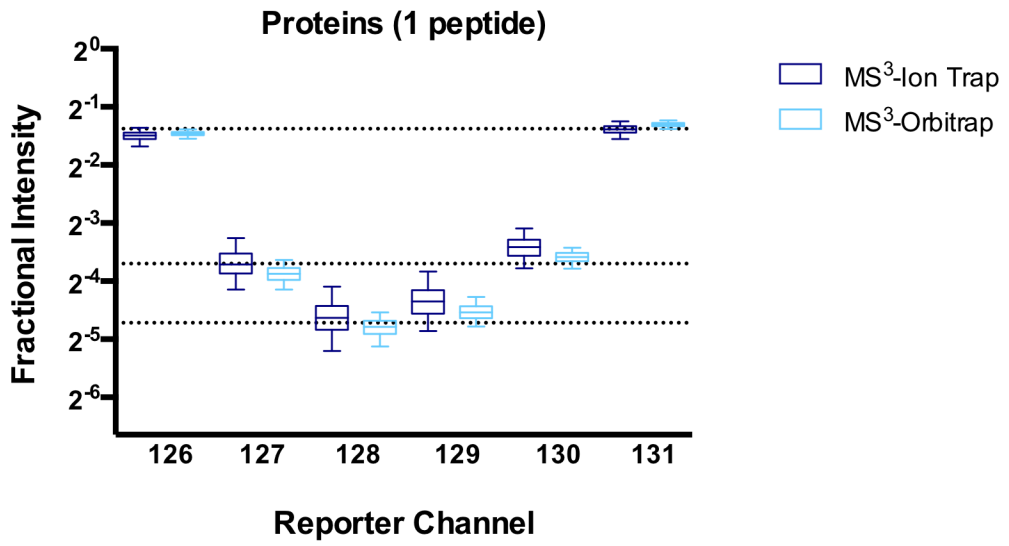

Figure S4. Accuracy and precision of protein quantification when collecting the MS^3-HCD scan using the linear ion trap (dark blue, left) or the Orbitrap (light blue, right). The proteins analyzed here were all identified by only 1 peptide that was quantified in all six reporter channels; see the main text for a similar analysis for proteins identified by >1 peptide. Box plots indicate the median (middle line), the interquartile range (box) and the 5^{th} and 95^{th} percentile for each experiment (which includes five technical replicates). The dashed lines indicate the values for the idealized fractionalized intensities.

Table S1. Quantification Data when Varying AGC Target Values in the Linear Ion Trap (MS^3-HCD Scan).

AGC Target	Median % Error (PSM)	Median % Error (Proteins)	Proteins[a]
1.0e3	6.89	5.15	1,632
5.0e3	5.05	4.46	1,395
1.0e4	4.83	4.43	1,335

[a] Number of proteins that were identified by at least 1 peptide that was quantified in all reporter channels. The data presented here is from one analysis, each, for the three different AGC targets tested.

Table S2. Number of PSM as a Function of Reporter Channels.

	MS³-Ion Trap		MS³-Orbitrap	
Number of Channels	**PSM[a] (Mean ± SD)**	**Percent of total PSM**	**PSM (Mean ± SD)**	**Percent of total PSM**
0[b]	7 ± 4	0.048	143 ± 4	1.4
1	8 ± 3	0.053	143 ± 22	1.4
2	15 ± 3	0.10	246 ± 18	2.3
3	39 ± 5	0.27	288 ± 19	2.7
4	118 ±15	0.81	582 ± 36	5.5
5	521 ±46	3.6	1,790 ± 26	17
6	13,916 ± 226	95	7,299 ± 226	70
Total	14,624 ± 243		10,490 ± 186	

[a] Number of peptide-to-spectrum matches (PSM) that had reporter ion intensities quantified in the corresponding number of channels. The average and standard deviation of five technical replicates are provided.

[b] MS^2 scans that either did not trigger an MS^3 scan or resulted in an MS^3 scan with no quantified reporter ions result in a PSM that was quantified in 0 channels.

Table S3. Protein Identification Rates.[a]

MS^3 Scan	Proteins Identified[b]	Proteins Quantified[c]	Proteins Quantified >1 peptide[d]
Ion trap	2,295 ± 38	2,229 ± 34	1,602 ± 32
Orbitrap	1,726 ± 18	1,342 ± 25	933 ± 17

[a] The average and standard deviation of five technical replicates are provided.
[b] Number of proteins that were identified by at least 1 peptide.
[c] Number of proteins that were identified by at least 1 peptide that was quantified in all reporter channels.
[d] Number of proteins that were identified by at least 2 peptides that were quantified in all reporter channels.